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TITLE: Identifying Metastasis Susceptibility Genes for Estrogen Receptor-Negative Breast Cancer

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14. ABSTRACT
The goal of this project is to identify metastasis susceptibility genes for estrogen receptor-negative breast cancer. Genetic analysis in mouse models has localized candidate genes to the distal end of mouse chromosome 6. Combined genomic and in vivo analysis has identified at least three candidate genes in this that are associated with metastatic progression in the mouse. Preliminary human association studies provide suggestive support for at least one of the candidate genes. Furthermore, evidence accumulated to date suggest that the effects on metastasis may be tumor-autonomous for at least some of the genes in question. To gain further information regarding additional candidate genes full genome sequencing has been performed on the MOLF/Ei strain of mice. In depth analysis of the polymorphic content of this strain may provide additional candidate genes for analysis as well as greater insight into the potential causative polymorphisms association with metastatic susceptibility.

15. SUBJECT TERMS
Breast cancer, metastasis, ER-negative, mouse models, genetic susceptibility, prognosis

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Introduction

Estrogen receptor-negative (ER-) tumors have the poor prognosis compared to ER+ breast cancer subtypes [1]. Mortality from ER- breast cancers, as with all solid tumors, is primarily due to sequela associated with metastatic lesions, rather than the primary tumor [2]. Despite of this, relatively little is understood regarding the mechanisms and origins of metastatic breast cancer. Better understanding of the etiology and mechanisms underlying metastatic disease would likely provide additional avenues for clinical intervention, particularly for ER- breast cancer for which no targeted therapies currently exist. The purpose of this project is to therefore investigate the factors contributing to metastatic disease in ER- breast cancer, using mouse genetically engineered cancer models and a meiotic genetic approach. Candidate genes identified by this screen are currently being validated in representative ER- mammary cancer mouse models, prior to evaluation in human data sets and cohorts. Successful completion of this project should provide additional etiologic information regarding the mechanisms of metastatic disease that may provide additional clinical intervention for the prevention or treatment of ER- breast cancer patients.

Keywords

Breast cancer, metastasis, ER-negative, mouse models, genetic susceptibility, prognosis

Overall Project Summary

Task 1: Identification of candidate metastasis susceptibility genes for ER- breast cancer (Hunter laboratory, NCI; months 1-12)

Tasks 1a-1c have been completed as anticipated. High quality RNA was isolated from the polyoma middle T (PyMT)-induced mammary tumors. Gene expression analysis was performed by submitting the PyMT mammary tumor RNA to the NCI Laboratory of Molecular Technology core facility, where it was arrayed on the Affymetrix Mouse ST 1.0 array. Expression data was provided to the Hunter laboratory who subsequently analyzed the tumor expression data for genes associated with metastatic disease. A total of 19 candidate genes were identified for further analysis in Task 2.

Task 1d was also completed during the proposed time period. Sequencing analysis of the Welcome Trust Sanger Centre Mouse Sequence Database was performed to identify those genes that contained amino acid substitutions. A total of 67 genes were identified, based on comparisons between the CAST/Ei strain, the closest related sequenced strain to MOLF/Ei, and FVB/NJ, the companion strain in the mouse cross used for this research proposal. These genes were subsequently analyzed for possible associations with human breast cancer progression under Task 3 (see below). To provide direct identification and analysis of putative functional polymorphisms in the MOLF/Ei strain, whole genome sequencing has been performed at 40x

coverage. Identification of polymorphisms in the MOLF/Ei genome is currently underway in collaboration with Dr. Thomas Keane at the Welcome Trust Sanger Centre.

Task 2: Screening mouse candidate genes for correlations with metastasis free survival using retrospective gene expression meta-analysis (Hunter laboratory, NCI; months 6-14)

The 19 genes whose expression was associated with metastatic disease in the mouse gene expression analysis were screened for the association of gene expression and distant metastatic disease in human gene expression data set using the Gene expression-based Outcome for Breast cancer Online (GOBO) [3] tool. This tool permits the meta-analysis of more than 1800 human breast cancer samples that were arrayed on the Affymetrix U133A chip. Each of the 19 candidate genes was screened to determine whether expression levels were correlated with distant metastasis free survival (DMFS) in patients stratified for ER status. No data was available for 6 of the 19 candidate genes (*C12orf72, KLHDC5, FGFROP2, LRRN1, IL17RE, CLEC6A*). Of the remaining genes, 3 (*C12orf35, ARNTL2, EMP1*) had significant gene expression correlations with DMFS in the ER$^-$ GOBO data sets and were considered for further analysis (Figure 1).

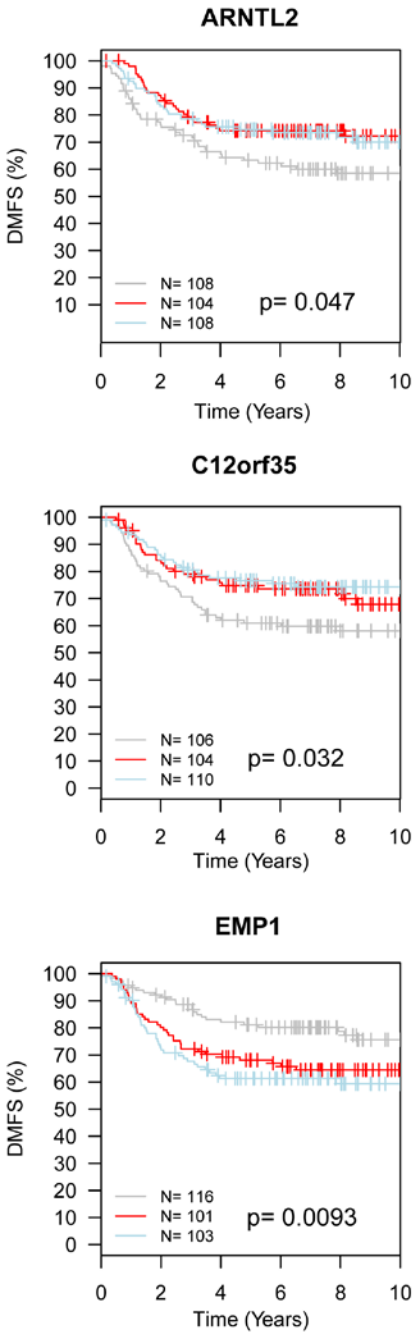

Figure 1: Kaplan-Meier analysis of candidate genes in the ER- samples of the GOBO meta-data analysis tool.

Task 3: Candidate genes identified in task 1 will be screened in a retrospective breast cancer cohort for associations with distant metastatic disease (Shu laboratory, Vanderbilt University; months 6-18).

The 67 genes with potential amino acid substitutions and the three genes displaying correlations with DMFS in the expression data were analyzed on the Vanderbilt breast cancer cohort. Genetic polymorphisms for each of the genes were examined for their association with disease free survival (DFS) and overall survival (OS) with adjustment for age at diagnosis, TNM stage and cancer treatments. A number of SNPs were significantly associated with DFS or OS but none of them retained a statistical significance after correction for multiple testing. Based on these results, *in vivo* modeling of candidate genes is being performed in the mouse prior to further investigation of this suggestive human association data. SNPs with suggestive associations in

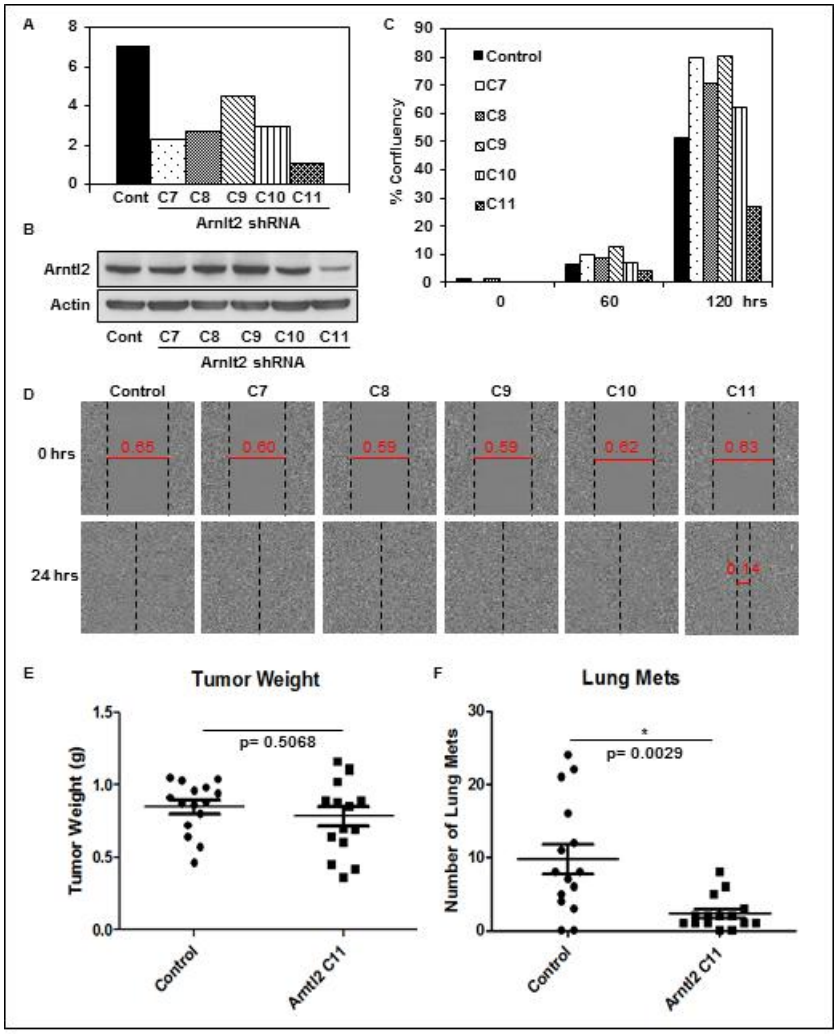

Fig 2. Effect of loss of Arntl2 expression *in vitro* and *in vivo*
(A) Arntl2 mRNA expression in 4T1 cells with control (scramble) and Arntl2 shRNA constructs. (B) Arntl2 protein expression. Actin was used as a loading control. (C) Cell proliferation of control and Arntl2 knock-down cells as measured by percent (%) confluency over five days. (D) Migration ability of control and Arntl2 knock-down cells. Wound was measured using arbitrary units at 0 hrs and 24 hrs. (E) Primary tumor weight of orthotopically injected Balb/c mice. 15 control and 15 Arntl2 C11 shRNA cells were injected. (F) Pulmonary nodules were grossly counted.

the discovery cohort whose associated genes are significant in the *in vivo* metastasis assays will be considered for genotyping in the human validation cohort in future studies.

Task 4: Validation of DMFS-associated SNPs in the validation cohort (months 18-30)

As indicated above in Task 3, work on this task has not yet been initiated.

Task 5: *In vivo* modeling of metastasis susceptibility genes (Hunter laboratory, NCI; months 6-36)

Task 5a&b: Knockdown cell lines have been generated for four candidate genes, *Arntl2* (Figure 2A), *2810474O19Rik* (*C12orf35*) (Figure 3A+B), *4833442J19Rik* (*C12orf72*), and *Emp1*. *2810474O19Rik*, *4833442J19Rik* and *Arntl2* were selected because they reside under the maximal peak of association with DMFS in the mouse cross. In addition, these genes demonstrate the strongest association with DMFS in the mouse gene expression data. Furthermore the human orthologs of *2810474O19Rik* and *Arntl2* correlate with DMFS in retrospective human gene expression data. Expression data for the human ortholog for *4833442J19Rik* was not available and therefore could not be evaluated. *Emp1* was also selected due to a significant association with DMFS in the genetic data, the human gene expression data and a suggestive association with an enhancer SNP in the first intron in the human discovery cohort data (Task 2, data not shown).

Orthotopic implantation of the knockout cell lines was performed and the effect on pulmonary metastasis evaluated. No significant difference was observed between the control

and knockdown cell lines for *4833442J19Rik* in the preliminary experiment. Further work on this gene was therefore discontinued (data not shown). Significant associations between metastatic disease were observed for knockdowns for *2810474O19Rik*, *Arntl2* and *Emp1* (Figure 2 and 3). Over-expression of *2810474O19Rik, Arntl2 and Emp1* cell lines is currently in progress.

Task 5c: Importation and colony establishment for genetically engineered models of *Arntl2* and *2910474O19Rik* have been established in the Hunter laboratory animal colonies. Both models were obtained from the KOMP Project resources, as planned. Importation of *Emp1* models will be considered after the over-expression studies have been completed.

Task 5d: Experimental crosses between the *Arntl2* and *2810474O19Rik* genetically engineered models and the MMTV-PyMT model is currently in progress. Preliminary evidence suggests that constitutional reduction of *2810474O19Rik* levels significantly influences metastatic progression of the MMTV-PyMT induced mammary tumors (Figure 4). At this time, the *Arntl2* cross does not have sufficient data to make any preliminary evaluation. Additional animals are required for both crosses for conclusive analysis.

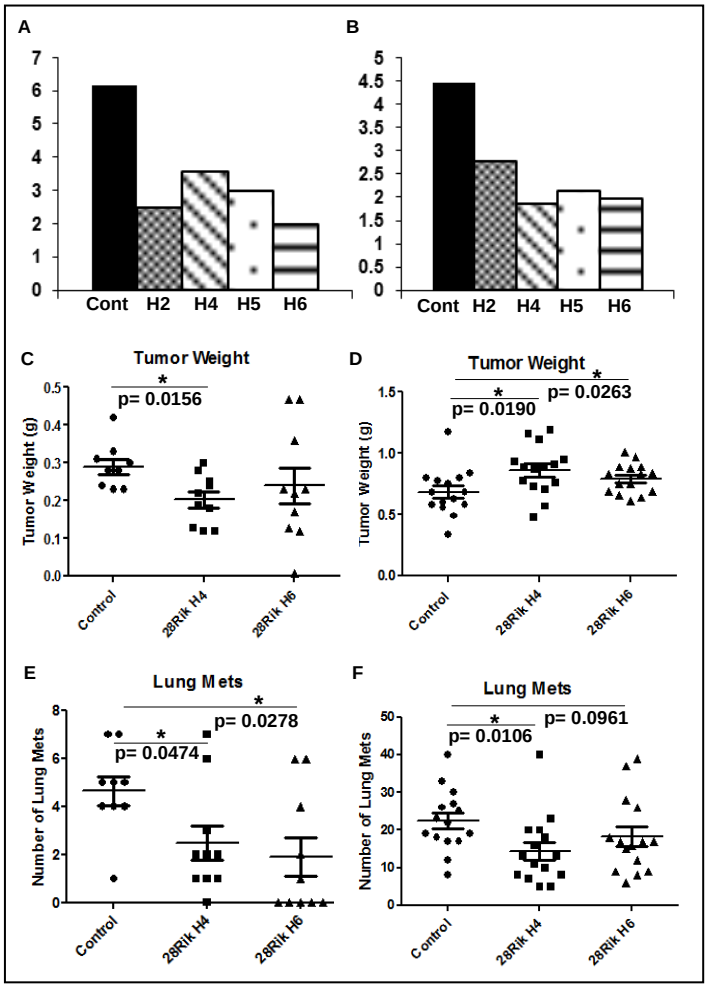

Fig 3. Tumor-autonomous effect of *2810474O19Rik in vivo*.
(A+B) Knock-down effect of four independent shRNA in Mvt-1 (A) cells and 6DT1 (B) as measured by qPCR.
(C+D) Primary tumor weight of Mvt-1 (C) and 6DT1 (D) cells
(E+F) Number of lung metastases upon orthoptopic injection of Mvt-1 (E) and 6DT1 (F) cells.

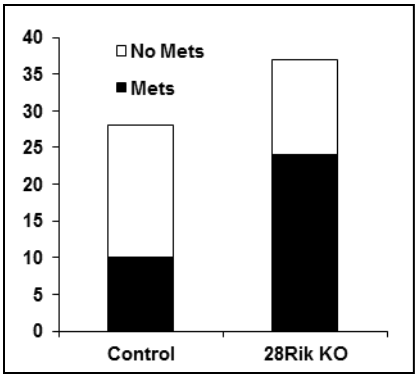

Fig 4. Stroma-dependent effect of *2810474O19Rik in vivo*.

Task 5e: Orthotopic implantation of unmodified tumor cell lines into mice carrying a knockout of *2810474O19Rik* heterozygously does not demonstrate any difference when compared to wildtype control animals (Figure 5). This suggests that the effect of *2810474O19Rik* on metastasis is a tumor-autonomous effect, rather than a stromal effect or a combination of a stromal and tumor effect. Similar

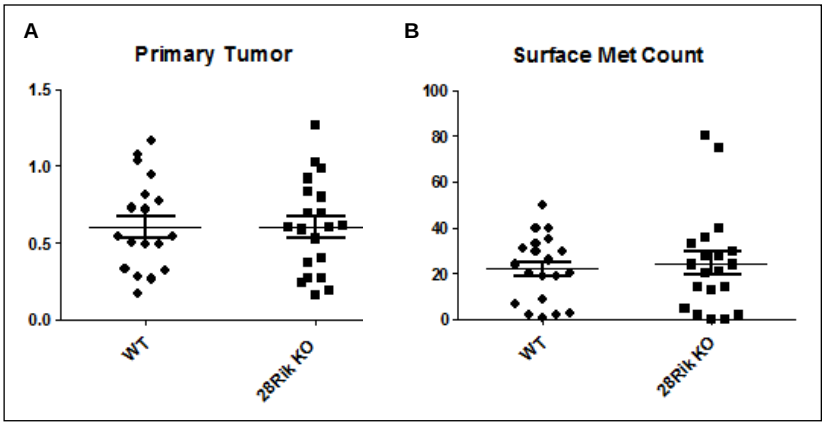

Fig 5. Stromal effect of *2810474O19Rik* in a mouse model of breast cancer. (A) Primary tumor weight of injected cells. Tumors were measured 28 days post-injection. (B) Surface metastasis count of the lungs from (A).

experiments for *Arntl2* also demonstrated no difference indicating that the effect of *Arntl2* is also a tumor-autonomous effect (Figure 6).

Task 5f: Genome editing experiments have not yet been initiated at this time.

Key Research Accomplishments

- Identification of *2810474O19Rik, Arntl2* and *Emp1* as potential metastasis susceptibility genes in a model of ER- breast cancer
- *In vivo* validation of *2810474O19Rik, Arntl2* and *Emp1* as metastasis susceptibility genes, as demonstrated by gene knockdown experiments in models of ER- human breast cancer.

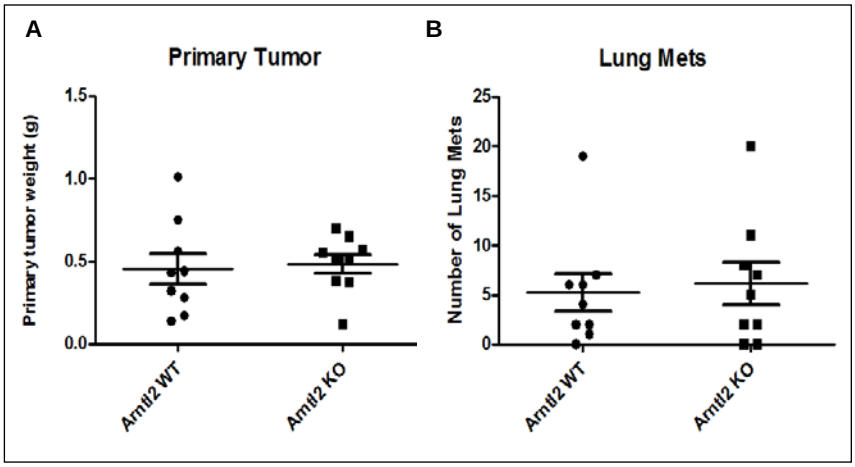

Fig 6. Stromal effect of *Arntl2* in vivo. (A) Primary tumor weight of orthotopically injected 4T1 cells into Arntl2 WT and KO mice. (B) Surface lung metastasis count of the injected mice in (A).

- Demonstration that *2810474O19Rik* and *Arntl2* exert their metastasis-associated effects through tumor-autonomous mechanism.
- Complete genome sequence of the MOLF/Ei inbred mouse strain at 40x coverage

Conclusions

The data gathered to date suggest that multiple genes contribute to susceptibility to metastasis potential for ER- breast cancer. Identification and characterization of these genes and their mode of action in breast cancer progression will lead to novel insights and potentially point toward new therapeutic strategies for this class of breast cancer. In the future the Hunter

laboratory will continue to explore the role of these genes in breast cancer by a combination of *in vitro* and *in vivo* methods to refine our understanding of the mechanisms involved and the pathways that play important roles in tumor progression. These efforts will consist of experimental crosses between knockout strains and the MMTV-PyMT model, as described in task 5, as well as investigations into whether alterations of these genes affect any of the intermediate processes in the metastatic cascade such as invasion, migration, apoptosis, anoikis etc.

Publications, Abstracts and Presentations

Presentation at the 27th International Mammalian Genome Conference, Salamanca, Spain "A metastasis susceptibility gene for estrogen receptor negative breast cancer maps to the distal end of chromosome 6" Ngoc-Han Ha, Ying Hu, Mia Williams, Rosan Nieves and Kent Hunter

Inventions, Patents and Licenses

Nothing to report

Reportable Outcomes

Nothing to report

Other Achievements

Nothing to report

References

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Appendices

None